L-Arginine and L-Canavanine Metabolism in Jack Bean, *Canavalia ensiformis* (L.) DC. and Soybean, *Glycine max* (L.) Merr.[1]

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ABSTRACT

Studies have been conducted with the arginase (L-arginine amidino-hydrolase, EC 3.5.3.1) of two legumes: jack bean, *Canavalia ensiformis* (L.) DC., a L-canavanine-containing plant and soybean, *Glycine max*, a canavanine-free species. Analyses of the arginase obtained from gradient-purified mitochondria of these legumes revealed that the arginine-dependent (ADA) and canavanine-dependent activities (CDA) were localized within this organelle.

Kinetic analyses of affinity-purified mitochondrial arginase revealed an apparent K_m of 7 to 8 millimolar for arginine with both the jack bean and soybean arginases. Comparable determinations with canavanine revealed an apparent K_m of 38 millimolar with the jack bean enzyme; the affinity for this arginine analog with the soybean enzyme is so poor that product formation remained linear even with a canavanine concentration of 890 millimolar.

A single macromolecule appears to be responsible for both the ADA and CDA of jack bean arginase. Ion-exchange chromatography of mitochondrial arginase revealed that the ADA and CDA eluted as a single, discrete peak from DEAE-cellulose. Analyses with arginine- and canavanine-linked Sepharose failed to reveal more than one enzyme. Both the ADA and CDA increased by nearly identical amounts following elution from arginine- and canavanine-linked cyanogen bromide-activated sepharose. Neither ADA nor CDA increased preferentially over the other.

Arginase (L-arginine amidinohydrolase, EC 3.5.3.1) catalyzes the hydrolysis of L-arginine to L-ornithine and urea. In many leguminous plants, L-canavanine, the guanidinooxy structural analog of L-arginine, serves as an important nitrogen-storing metabolite (1, 14). These plants presumably employ arginase to hydrolyze L-canavanine to L-canaline and urea (7, 12).

This study compared the catalytic action with regard to arginine and canavanine of the arginase from two legumes: jack bean, *Canavalia ensiformis* (L.) DC., a canavanine-containing plant and soybean, *Glycine max* (L.) Merr. var Williams, a canavanine-free species (15). It sought to determine whether a single macromolecule fostered the catabolism of L-arginine and L-canavanine and to better understand the nature of these cata-bolic reactions. This work commenced a series of biochemical studies designed to provide greater insight into the metabolic relationship between the primary metabolites, L-arginine and its ornithine-urea cycle allies, and their nonprotein amino acid analog counterparts.

MATERIALS AND METHODS

Plant Materials. Jack bean and soybean plants were grown either in a greenhouse under natural illumination from April to August or in a controlled environmental chamber (Percival, model 103) at 30°C day and 20°C night temperature with a 16/8 h light-dark regime; the RH was 90%.

Fractionation of Cotyledon Homogenates. Cotyledons from 8- to 10-d-old plants were homogenized in 1 volume of grinding medium (300 mM mannitol, 25 mM MOPS[3] [pH 7.5], 1 mM $MnCl_2$, 4 mM cysteine, 0.2% BSA [w/v]) at 4°C using a Braun juicer. The homogenate, passed through two layers of Miracloth, was centrifuged at 1,500g (5min), 12,000g (20 min), and 30,000g (30 min). The pellets were suspended in wash medium (300 mM mannitol, 25 mM MOPS [pH 7.2], 1 mM $MnCl_2$, 0.2% BSA [w/v]), solubilized with 1% Triton X-100, and assayed for enzymic activity.

Isolation of Cotyledon Mitochondria. Mitochondria were isolated by the procedure of Douce *et al.* (8) with several modifications. The initial low-speed centrifugation was reduced to 1,500g for 5 min. The supernatant solution was centrifuged at 12,000g for 20 min and the crude mitochondrial fraction (pellet) was suspended in wash medium. Mitochondria were washed by centrifuging at 1,500g (10 min), pelleted at 11,000g (15 min), and suspended in approximately 4 ml of wash medium.

Gradient Centrifugation. Washed mitochondria were layered onto step gradients consisting of 13.5%, 21%, and 45% (v/v) Percoll. In addition, each Percoll solution contained 350 mM sucrose, 0.2% (w/v) BSA, and 10 mM MOPS (pH 7.2) after the method of Jackson *et al.* (9). $MnCl_2$ (1 mM) was added to each Percoll solution to maintain arginase activity. Mitochondria migrated to the 21 to 45% Percoll interface following centrifugation at 7,500g for 30 min. The overlying solution was removed by aspiration, the organelles suspended in wash medium lacking BSA, and then pelleted at 13,000g for 15 min. Mitochondria were suspended either in wash medium for respiration assays or solubilized with 1% Triton X-100 in 25 mM MOPS (pH 7.2), 1 mM $MnCl_2$, and 1 mM DTT for enzyme analysis.

Mitochondrial Respiration. Mitochondrial respiration was measured polarographically with a Clark electrode (6).

Enzyme Purification. Proteins from solubilized mitochondria

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[3] Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; ADA, arginine-dependent activity; CDA, canavanine-dependent activity.

were precipitated with solid, enzyme-grade ammonium sulfate. The 30 to 60% fraction was collected by centrifugation (10,000g for 15 min), and suspended in 10 mM MOPS (pH 7.5) that contained 1 mM each of $MnCl_2$ and DTT. Desalting and protein concentration was accomplished by ultrafiltration on an Amicon P-10 membrane.

Affinity chromatography was employed to isolate the mitochondrial protein(s) that catalyzed the hydrolysis of arginine and canavanine. Arginine-linked Sepharose was obtained commercially (Pharmacia) while canavanine-linked Sepharose was prepared from cyanogen bromide-activated Sepharose 4B (Pharmacia). Mitochondrial protein in a minimum volume of buffer (10 mM MOPS [pH 7.5] and 1 mM $MnCl_2$) was loaded onto affinity columns (0.5 × 5 cm) equilibrated with 10 mM Tricine (pH 7.7). The columns were washed with 10 mM Tricine (pH 7.7) and the hydrolytic activity was removed by elution with 125 mM glycine/KOH (pH 9.7) or 125 mM Tricine (pH 7.7) for protein retained on arginine-linked Sepharose or canavanine-linked Sepharose, respectively.

Enzyme Assays. ADA was measured by monitoring ornithine production (5) while CDA was determined by measuring canaline formation (after conversion to *O*-ureidohomoserine) (13). ADA and CDA were determined at their respective pH optima (pH 9.7 and 7.7) in a final volume of 1 ml. Arginine-dependent reactions were carried out in 50 mM glycine/KOH (pH 9.7) containing 2100 mM arginine (pH 9.7), 1 mM DTT, 1 mM $MnCl_2$, and 10 μg protein. Canavanine-dependent reactions were conducted in 50 mM Tricine (pH 7.7) containing 350 mM canavanine (pH 7.7), 1 mM DTT, 1 mM $MnCl_2$, and 10 μg protein. Enzymic activity was maximized by preincubating the protein with $MnCl_2$ for 30 min at 37°C prior to substrate addition. Enzyme assays, allowed to proceed for 30 min at 37°C, were terminated with 7% (v/v) $HClO_4$ and neutralized with 3.3 N KOH. Protein concentrations were determined by the method of Bradford (4) using BSA (fraction V) as the standard.

Inhibition Studies. Inhibition of ADA and CDA by canavanine and arginine, respectively, were conducted at pH 7.7, since canavanine was such a poor substrate at pH 9.7 that it did not substantially inhibit ADA. Reactions were conducted in the presence of apparent K_m levels of either arginine (8 mM) or canavanine (38 mM). One to 10 times the apparent K_m level of each inhibitor was added to appropriate reaction mixtures to determine the degree of inhibition. ADA inhibition by canavanine was monitored by ornithine formation while CDA inhibition by arginine relied upon canaline production. The enzyme (10 μg protein, step 4) was preincubated at 37°C for 30 min in the presence of 1 mM $MnCl_2$ and DTT prior to the simultaneous addition of substrate and inhibitor. Assays, conducted for 30 min, were terminated with 7% (v/v) $HClO_4$ as described previously.

RESULTS

Subcellular Localization of Hydrolytic Activity. The subcellular distribution of the enzymes that catalyze arginine and canavanine hydrolysis in jack bean and arginine in soybean was determined by differential centrifugation. Partitioning of ADA and CDA from jack bean is shown in Table I. The highest specific activities for hydrolysis of both substrates occurred in the mitochondrial fraction (12,000g pellet). The soluble or cytoplasmic fraction (30,000g supernatant solution) also contained a substantial portion of the total activity; however, the specific activity of this fraction was extremely low (less than 15% of the 12,000g fraction). Arginase activity in this fraction may result from a cytoplasmic isozyme or simple release of mitochondrial enzymes during homogenization and/or centrifugation. The distribution of arginase activity from soybean cotyledons was similar to that found for jack bean (data not provided).

Table I. *Subcellular Distribution of Arginine-Dependent and Canavanine-Dependent Hydrolytic Activity from 8-Day-Old C. ensiformis Cotyledons*

Fraction	Protein	Total Activity		Specific Activity		Yield	
		ADA	CDA	ADA	CDA	ADA	CDA
	mg	*nkat*		*nkat/mg*		*%*	
Homogenate	6,250	11,250	1,875	1.8	0.3	100	100
1,500g pellet	270	1,540	297	5.7	1.1	14	16
12,000g pellet	450	4,275	810	9.5	1.8	38	43
30,000g pellet	240	120		0.5		1	
30,000g super.	5,000	5,000	750	1.0	0.15	44	40

The localization of arginase within the mitochondria has been shown for several plants (2, 10, 19, 20). Gradient-purified mitochondria from jack bean and soybean were examined to determine whether ADA was similarly compartmentalized, and if CDA was also associated with this organelle. The mitochondria obtained were metabolically active and typically oxidized succinate, NADH, and malate with respiratory control ratios ranging from 4 to 6 in the most active preparations. Approximately 40% of both the total ADA and CDA were retained within the jack bean mitochondria (Table II). Moreover, the specific activity of both the ADA and CDA increased nearly 60-fold by isolation of the organelles compared to cotyledon homogenates. This suggested that the enzyme(s) responsible for hydrolysis of both amino acids resided within the mitochondria. The recovery and specific activity of the arginase from soybean mitochondria was not as great as that from jack bean mitochondria (Table III). This finding may reflect difficulties in maintaining organelle integrity during the isolation procedure (11) that were not encountered with jack bean preparations.

The possibility that other enzymes involved with arginine metabolism might occur in jack bean and soybean mitochondria was examined. Enzyme assays for ornithine carbamoyltransferase, argininosuccinic acid synthetase, and argininosuccinic acid lyase revealed no appreciable activity in gradient-purified mitochondria from either legume.

Partial Purification and Characterization of Arginase. Triton-solubilized mitochondrial protein was precipitated with solid, enzyme-grade $(NH_4)_2SO_4$ before affinity chromatography on arginine-linked sepharose. The specific activity of both ADA and CDA increased more than 15-fold after affinity chromatography as compared to gradient-purified materials (Table II). This resulted in a purification of more than 900-fold relative to the original cotyledon homogenate. SDS-gel electrophoresis of the affinity purified jack bean enzyme revealed one major and four minor bands.

Comparative kinetic analyses of the ADA and CDA of jack bean and soybean were conducted with the affinity-purified protein. The enzymes responsible for ADA in both legumes revealed a comparable affinity for arginine (apparent K_m of 7 to 8 mM); their relative affinities for canavanine were vastly different. The jack bean enzyme was found to have an apparent K_m of 38 mM for canavanine. Soybean, on the other hand, possessed a canavanine K_m that was much higher. In fact, canaline formation remained linear even when the canavanine concentration reached 890 mM, the maximum substrate concentration possible under our assay conditions. This precluded determination of the apparent K_m of soybean CDA for canavanine and illustrates clearly the miniscule affinity of the soybean enzyme for this arginine analog.

Chromatographic analysis of mitochondrial protein from Triton-solubilized, gradient-purified mitochondria with arginine- and canavanine-linked Sepharose revealed that neither ADA nor CDA increased preferentially over the other (Table III). Both

Table II. *Purification of Arginine-Dependent and Canavanine-Dependent Activity from 8-Day-Old Cotyledons of C. Ensiformis and G. max*

Fraction	Protein	Total Activity		Specific Activity		Yield		Purification	
		ADA	CDA	ADA	CDA	ADA	CDA	ADA	CDA
	mg	*nkat*		*nkat/mg*		*%*		*—fold*	
C. ensiformis									
1. Homogenate	4,125	13,612	2,887	3.3	0.7	100	100	1	1
2. Washed mitochondria	88	12,760	2,552	145	29	94	88	48	41
3. Gradient-purified mitochondria	32	5,984	1,312	187	41	44	45	62	59
4. Ammonium sulfate ppt.	1.9	1,360	298	715	157	10	10	138	224
5. Arg-linked Sepharose	0.2	622	130	3,100	650	5	5	942	930
G. max									
1. Homogenate	2,340	30,420		13		100		1	
2. Washed mitochondria	270	9.450		35		31		3	
3. Gradient-purified mitochondria	60	4,080		68		13		5	
4. Ammonium sulfate ppt.	4	632		158		2		12	
5. Arg-linked Sepharose	0.5	485		970		1.6		75	

Table III. *Separation of Arginine-Dependent and Canavanine-Dependent Hydrolytic Activity by Affinity Chromatography*

Fraction	Specific Activity		
	ADA	CDA	ADA:CDA
	nkat/mg		
Gradient-purified mitochondria	118	25	4.7:1
Arginine-linked Sepharose	732	170	4.3:1
Canavanine-linked Sepharose	624	147	4.3:1

Table IV. *Inhibition of C. ensiformis Arginine-Dependent and Canavanine-Dependent Hydrolytic Activity by Canavanine and Arginine, Respectively*

Arginine Concn.	CDA Inhibition[a]	Canavanine Concn.	ADA Inhibition[b]
MM	*%*	*mM*	*%*
8	5	38	3
20	20	76	18
40	28		
60	40		
80	48		

[a] Assays run in the presence of 38 mM canavanine (apparent K_m level).
[b] Assays run in the presence of 8 mM arginine (apparent K_m level).

activities increased by nearly identical amounts (6.2- versus 6.8-fold purification) following elution from arginine-linked Sepharose; similar results were obtained from canavanine-linked Sepharose (5.3 versus 5.9). The correspondence between the specific activity ratios (ADA:CDA) after elution from both affinity columns strongly suggested that hydrolysis of both arginine and canavanine in jack bean mitochondria was catalyzed by a single macromolecule.

Ion-exchange chromatographic studies also supported this conclusion. ADA and CDA eluted as a single, discrete peak from DEAE-cellulose (Whatman DE-52). The protein obtained following chromatography was purified over 300-fold. Kinetic studies of the DEAE-cellulose-treated enzyme revealed that the catalytic properties of the enzyme were altered. The apparent K_m for ADA increased nearly 3-fold to about 20 mM. Likewise, the apparent K_m for CDA increased from 38 mM to over 80 mM. For this reason, arginase purified by this method was not studied further.

Inhibition. The ability of canavanine to inhibit jack bean ADA or arginine to inhibit CDA at pH 7.7 was examined to determine the extent each substrate interfered with the hydrolysis of the other in vitro (Table IV). Canavanine was found to inhibit ADA at pH 7.7 (18% inhibition with 76 mM canavanine), but it did not inhibit ADA at pH 9.7. Arginine inhibited CDA at pH 7.7 (48% at 80 mM arginine) much more than canavanine interfered with ADA. In a similar vein, at a constant substrate concentration of 100 mM, at pH 7.7 canavanine and arginine generate a comparable amount of urea. At pH 9.7, however, urea production from canavanine is only 7% of that from arginine.

These results may reflect the charge state of the guanidino/oxyguanidino group on these amino acids since they possess different pK_a values. The guanidino group of arginine is fully protonated at pH 7.7 (18) while the guanidinooxy group of canavanine is uncharged at pH 9.7 (3). Arginine's capacity to inhibit CDA at pH 7.7 and function as an active arginase substrate at both pH values, may reflect the protonation of the guanidino group. At pH 9.7, where canavanine is deprotonated, it is a relatively poor substrate for arginase and has limited ability to inhibit ADA.

DISCUSSION

Arginase has been shown to mediate the hydrolysis of both L-arginine and L-canavanine in the canavanine-containing plant, *Canavalia ensiformis*. On the other hand, the arginase of the canavanine-free legume, *Glycine max* exhibits little demonstrable activity with canavanine. The experimental evidence supports strongly the existence of a single macromolecule in *C. ensiformis* that catalyzes the cleavage of both amino acids.

At a pH of 9 or above, the in vitro pH optima range for arginine, canavanine is virtually fully deprotonated. The anionic canavanine species is a very poor substrate for arginase and possesses little capacity to inhibit arginase-mediated cleavage of arginine. At physiological pH, canavanine exists in the cationic form and it is both an effective substrate for arginase and able to inhibit arginase-directed hydrolysis of arginine.

The existence of a single protein that fosters the catabolism of both arginine and canavanine gains added significance by focusing on the means whereby higher plants, by modification of the enzymes of primary metabolisn, may have evolved the biosynthetic ability to produce secondary metabolites. The arginase of jack bean, relative to that of soybean, is markedly superior in its

catalytic action with canavanine. Recent studies of the bruchid bettle, *Caryedes brasiliensis*, a canavanine-adapted, seed-predator revealed the evolution of an arginyl tRNA synthetase able to distinguish between arginine and canavanine (16). Canavanine-sensitive insects studied to date do not produce such a discriminatory enzyme. This discriminatory capacity may have conferred a general resistance to the incorporation of nonprotein amino acid analogs into proteins (17). It is germane to ask whether the arginase of *C. ensiformis* has been modified so as to accomodate canavanine? It may well be that, in canavanine-producing plants such as jack bean, the active site of existing enzymes functioning in the anabolism of primary metabolites was modified in order to produce secondary nonprotein amino acids that improved the overall Darwinian fitness.

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